Supplemental oxygen controls cellular proliferation and anastomotic intimal hyperplasia at a vascular graft-to-artery anastomosis in the rabbit

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Purpose: **The purpose of this study was to determine whether the administration of 40% supplemental oxygen (O_2) will decrease cellular proliferation and intimal hyperplasia (IH) at a prosthetic vascular graft (PVG)–to-artery anastomosis.**
Methods: **Twenty New Zealand white rabbits underwent placement of a 3-mm polytetrafluoroethylene graft in their infrarenal aorta. Four groups of five rabbits were placed either in a normoxic (21%) environment or in a 40% supplemental O_2 environment for 7 or 42 days. Twenty-four hours before the rabbits were humanely killed for aortic graft harvest, BrDU (5-bromo-2′-deoxyuridine) was injected into the rabbits intraperitoneally. Image analysis (Bioquant) morphometrics were used to measure cells with BrDU staining and intimal areas at the distal anastomosis. Cellular proliferation is defined as positively staining BrDU cells divided by all cells in the artery wall. IH is reported as a ratio between the intimal area and the medial area to standardize the varying aortic size and degree of aortic fixation among rabbits. The Student *t* test was used to compare cellular proliferation and IH between control and O_2-treated rabbits.**
Results: **Cellular proliferation in the intima at 7 days was significantly reduced in the O_2-treated animals (1.7% ± 1%) versus the control animals (28.6% ± 3%) (*P* = .0001). The cellular proliferation in the intima at 42 days returned to preoperative levels in the O_2-treated group (0.15%) and in the control group (0.11%) (*P* = not significant). IH at 7 days was minimal, and no difference between the O_2-treated group (0.017 ± .006) and the control group (0.009 ± .03) (*P* = not significant) was found. IH was significantly reduced at 42 days in the O_2-treated animals (0.031 ± .012) when compared with the control animals (0.193 ± .043) (*P* = .006).**
Conclusions: **Supplemental O_2 (40%) significantly reduces cellular proliferation and IH at the distal anastomosis of a PVG-to-artery anastomosis in the rabbit model. (J Vasc Surg 2001;33:608-13.)**

Intimal hyperplasia (IH) is a pathologic response to vascular injury, which complicates vascular bypass graft procedures by causing stenoses, graft failures, and reoperation.[1] Because of the impact on quality of life, medical costs, and the potential loss of life and limb, researchers in several laboratories are actively examining the causes and mechanisms of IH. The formation of IH is generally thought to occur as a result of endothelial cell injury, vascular smooth muscle cell migration, cell proliferation, and extracellular matrix formation.[2]

The effective treatment and prevention of IH in clinical practice continue to elude vascular surgeons. Strategies, including pharmacologic therapy, antioxidant therapy, the local infusion of heparin, and gene-directed therapy of adenoviral vectors,[3-6] have yet to be widely used and accepted as methods for preventing IH. One possible impediment to the widespread use of the various therapies shown to be effective in in vitro and in animal studies is the expense and complexity in the practical utility of applying these therapies on a daily basis.

On the other hand, oxygen is widely available with a long history of relative safety. Only recently, short-term supplemental oxygen therapy has been found to prevent surgical wound infection after colorectal surgery. With such a widely available therapeutic modality, oxygen, as a preventive therapy for IH, has not fully been explored.

In 1944 Hueper[7] first suggested that artery wall hypoxia was involved in artery wall pathology. Since then, several others have independently demonstrated depressed partial pressure of oxygen (Po_2) levels in the artery wall when exposed to conditions known to cause atherosclerosis or IH.[8,9] Current research has shown that artery wall hypoxia occurs after placement of a prosthetic vascular graft (PVG)–to-artery anastomosis, and that this hypoxia is associated with increased smooth muscle cell proliferation.[10,11] The next appropriate step, then, is to determine whether the administration of supplemental oxygen could inhibit or decrease IH formation.

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We hypothesize that artery wall hypoxia is a key factor in causing IH through increased cellular proliferation at a PVG-to-artery anastomosis and that supplemental oxygen may prevent IH. The purpose of this study was to determine whether 40% supplemental oxygen treatment would decrease cellular proliferation and anastomotic IH at the distal anastomosis of a PVG-to-artery anastomosis in the rabbit.

METHODS

Animal model. Twenty rabbits, five in each group, were assigned to one of the four following arms of the study: 7-day control, 7-day supplemental oxygen, 42-day control, and 42-day supplemental oxygen. Immediately after placement of the PVG-to-artery anastomosis, the rabbits were assigned to a control cage (21% oxygen environment) or to an oxygen chamber (40% oxygen environment) designed and built by Plas Laboratories (Lansing, Mich). These chambers were deigned to regulate the environmental oxygen content (40%) and relative humidity (50%). New Zealand white rabbits were conditioned to their environment and fed standard rabbit chow and water as desired at least 2 weeks before the procedure. All rabbits tolerated the oxygen chambers well, and oxygen-supplemented rabbits were exposed to a normoxic environment (inspired oxygen: 21%) for 1 to 2 hours a day to allow for daily oxygen chamber cares such as food, water, and bedding changes. All animal procedures conducted during the study were in accordance with an animal use protocol approved by the Minneapolis Veterans Affairs Institutional Animal Care and Use Committee.

Placement of the PVG. After 2 weeks acclimatization, rabbits were anesthetized with ketamine (40 mg/kg) and xylazine (5 mg/kg) intramuscularly and blindly intubated with a 3-mm endotracheal tube. Rabbits then received 150,000 units of penicillin subcutaneously, a 24-gauge intravenous catheter was placed in a marginal ear vein, and the rabbits were placed on a water-circulating heating pad to maintain core body temperature. Inhalation anesthesia of 1% to 1.5% isoflurane was used to maintain rabbits under general anesthesia for the duration of the operation. Under 2.5×-loupe magnification and aseptic technique, a midline incision from the xiphoid bone to the pubis bone was used to expose the infrarenal aorta. The intestines were retracted to the right, and the peritoneum was dissected to free the distal 2 cm of aorta. The rabbits then received 250 U/kg of intravenous heparin sodium 3 minutes before cross-clamping of the aorta.

The infrarenal aorta, 2 cm proximal to the aortic bifurcation, was clamped with two bulldog clamps, and a longitudinal incision in the aorta was made with an 11-blade scalpel. The incision was lengthened to 3 mm in size with iris scissors. A prosthetic 3-mm polytetrafluoroethylene graft (W.L. Gore Associates, Inc, Flagstaff, Ariz), 3.5 cm in length, was then sewn in an end of graft to side of artery fashion with a running 7-0 Prolene suture. Once the proximal anastomosis was complete, the above steps were then repeated for the distal anastomosis. After graft placement, a 4-0 silk tie was placed just proximal to the distal anastomosis to ensure 100% blood flow through the graft, a functional end-to-end anastomosis.

BrdU administration and harvesting of the aortic graft. Twenty-four hours before the rabbits were humanely killed, BrdU (5-bromo-2′-deoxyuridine) (100 mg/kg; Sigma Chemical Co, St Louis, Mo) was administered intraperitoneally. Rabbits were then euthanized with an overdose of pentobarbital. The infrarenal aorta and the inferior vena cava were cannulated with 20-gauge catheters. Each rabbit was flushed with normal saline and was exsanguinated. The aorta and the aortic graft were perfusion fixed at 300 mm Hg of pressure with Karnovsky's solution for 10 minutes.[8] The aorta was harvested and prepared for histologic analysis. The slide specimens were then stained with hematoxylin and eosin, Verhoff-van Gieson, and elastin stains.

Sample processing and BrdU staining. The BrdU staining was performed according to the suggested procedures of the Zymed BrdU staining kit (Zymed Laboratories, San Francisco, Calif). Each specimen was embedded in paraffin, and 4-μm thick sections were cut on a microtome. The sections were cut transversely, 1 to 2 mm distal to the distal anastomosis, and longitudinally to demonstrate the lumen of the artery and the graft loop. Sections were mounted on glass slides and dried in a 60°C oven for 30 to 60 minutes. They were then deparaffinized in xylene and rehydrated with a series of ethanols. Endogenous peroxidase activity was quenched in peroxidase-quenching solution for 10 minutes, and the slides were rinsed in phosphate-buffered saline (PBS). Sections were treated with trypsin for 10 minutes in a moist chamber at 37°C. The sections were incubated with denaturing solution for 30 minutes to increase the accessibility of the antigen. The sections were treated for nonspecific labeling with blocking solution for 10 minutes. Biotinylated mouse anti-BrdU reagent was applied to the sections for 60 minutes at room temperature. The sections were rinsed again in PBS and incubated for 10 minutes with streptavidin-peroxidase reagent. After rinsing, the color was developed by incubation with DAB (3,3′-diaminobenzidine) mixture with the aid of a microscope. Sections were counterstained with hematoxylin, washed in tap water, transferred into PBS until the nuclei were blue (approximately 2-3 minutes), and then rinsed in distilled water. Slides were dehydrated in graded alcohol solutions, cleared in xylene, and cover slipped.

Image analysis (Bioquant) morphometric measurement of BrdU labeling and IH. Cells that incorporated BrdU were identified as having a distinctive brown pigment within their nuclei. A Zeiss microscope with a digitized camera attachment (Oberkochen, Germany) fed images directly into a personal computer–based microprocessor. Image analysis (Bioquant) morphometrics program was used to count all BrdU-labeled cells and all other cells within a microscopic field. This process was repeated until the entire circumference of the artery wall was evaluated. Cellular proliferation was defined as all cells positively staining for BrdU divided by all cells present

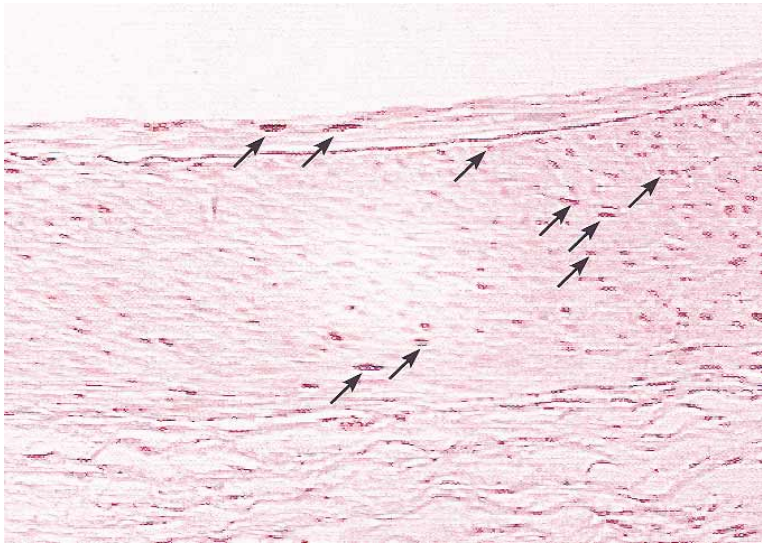

Fig 1. Cell proliferation in PVG-to-artery anastomosis. Immunoperoxidase staining of BrdU-labeled cells. Darkly stained nuclei (*arrows*) represent positive staining cells. Seven-day oxygen treated rabbit (Biotinylated mouse anti-BrdU, original magnification × 200).

Fig 2. Seven-day oxygen-treated rabbit (Biotinylated mouse anti-BrdU, original magnification ×200).

within the artery wall. Cellular proliferation is reported in percent units (positively stained BrdU cells/all cells).

The image analysis (Bioquant) morphometrics program was also used for measuring the intimal area (IA) to medial area (MA) ratio. The digital camera was calibrated by measuring a known distance on the microscope stage. The intimal and medial boundaries were then marked, and the IA and the MA were then automatically calculated. The standardization of IA to MA was performed because of varying animal aortic sizes and the extent of fixation.

Artery blood gas analysis. Arterial blood gases were obtained from the central ear artery while the rabbits were housed in the oxygen chambers to ensure proper chamber function. We could then document arterial blood oxygen content.

Artery wall oxygen tension measurements. Artery wall oxygen tensions were directly measured with a platinum-iridium microelectrode 2 mm distal to the distal anastomosis at 10-μm intervals with a chemical microsensor (Model 1201; Diamond General Corp, Ann Arbor, Mich). The measurement, 2 mm distal to the distal anastomosis, was performed because the degree of scarring makes preservation of the vasa vasorum unlikely. Also, if measurements were taken at the anastomosis, electrode

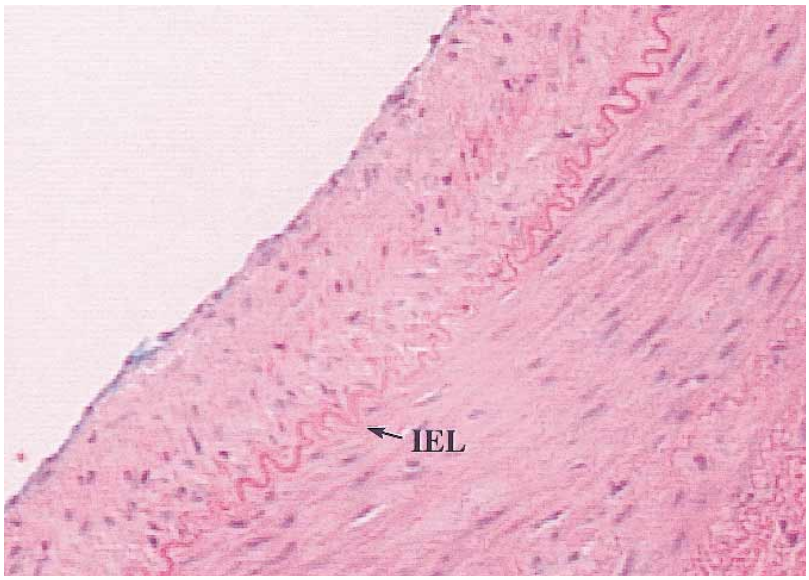

Fig 3. IH in PVG-to-artery anastomosis. A 42-day control rabbit (hematoxylin and eosin stain, original magnification ×200). *IEL*, Internal elastic lamina.

breakage would occur more frequently because contact with suture material would cause breakage.

Rabbits were exposed to either a 21% oxygen environment (control) or a 40% oxygen environment during artery wall oxygen tension measurements. The oxygen tension measurements followed a curvilinear pattern where the nadir was found to be two thirds internal to the adventitial layer. In this report, arterial Po_2 levels at the adventitial (outer) layers are presented to summarize the change in oxygen tension values at the artery anastomosis. Complete artery wall oxygen tension profiles at varying oxygen concentrations at different time points have been published elsewhere.[10,11]

Statistical analysis. All data were expressed as mean ± SEM. The Student 2-tailed *t* test with Excel 98 for the Macintosh computer (Microsoft, Redmond, Wash) was used to compare cellular proliferation and IH between the control rabbits and rabbits treated with supplemental oxygen. A *P* value less than .05 was considered significant.

RESULTS

During the follow-up period, no animals had complications of an occluded graft, which would be demonstrated by progressive paresis and subsequent paralysis. The Po_2 in the arterial blood gas analysis was 297 ± 17 mm Hg in the oxygen-treated animals and 87 ± 4 mm Hg in the control animals. Artery wall oxygen tension levels were directly measured 2 mm distal to the distal anastomosis. The results of artery wall tension levels at the adventitial layers demonstrated a nadir Po_2 level of 25.4 ± 2.6 mm Hg in the 7-day control rabbits and a nadir Po_2 level of 98.3 ± 9.3 mm Hg in the 7-day supplemental oxygen O_2-treated rabbits. The oxygen tension levels in the pretreated rabbits, where no graft anastomosis was placed, were 60 ± 2 mm Hg. A nadir Po_2 level of 25 ± 1.9 mm Hg was found in the 42-day control rabbits, and a nadir Po_2 level of 197 ± 10.3 mm Hg was found in the 42-day O_2-treated rabbit. Cellular proliferation at 7 days was significantly higher in the control animals, with 28.6% ± 3% (Fig 1), when compared with the oxygen-treated animals, with 1.7% ± 1% ($P = .0001$, Fig 2). The cellular proliferation rates were no different at day 42 and returned to preoperative levels with 0.13% cellular proliferation in controls compared with 0.15% cellular proliferation in the O_2-treated animals (P = not significant).

IH was no different between control animals with an IA/MA ratio of 0.017 ± .006 and O_2-treated animals with an IA/MA ratio of 0.009 ± .003 at day 7 (P = not significant). However, at day 42, a significant decrease in IA/MA ratio was found in the O_2-treated animals. Control animals had an IA/MA ratio of 0.193 ± 0.043 (Fig 3) compared with the O_2-treated animals, which had a ratio of 0.031 ± 0.012 ($P = .006$, Fig 4).

DISCUSSION

This is the first in vivo evidence that supplemental oxygen can control cellular proliferation and IH at a PVG-to-artery anastomosis. This finding is significant in that supplemental oxygen is a readily available treatment modality with a long history of relative safety. If IH can be controlled by such a simple treatment, significant morbidity, mortality, and health care resources can be saved in terms of preventing graft stenosis, occlusion, reoperation, and limb loss.

In previous work in our laboratory, we have demonstrated that the creation of a PVG-to-artery anastomosis

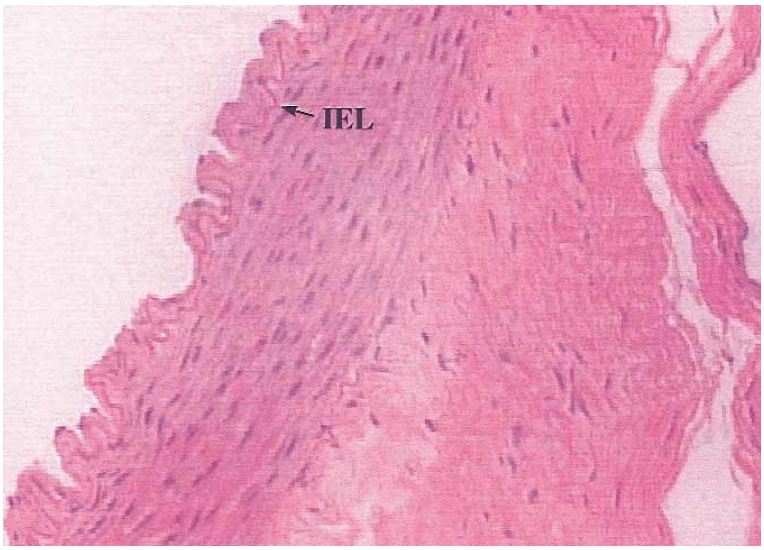

Fig 4. A 42-day oxygen-treated rabbit (hematoxylin and eosin, original magnification ×200). *IEL,* Internal elastic lamina.

results in significant artery wall hypoxia, which resolves in 42 days.[11] More important, after the first 7 days after PVG placement, the artery wall is most hypoxic, which correlated with significantly increased smooth muscle cell proliferation.[10] Although an association between artery wall hypoxia and smooth muscle cell proliferation was established, a cause and effect relationship between hypoxia and smooth muscle proliferation still needs to be elucidated.

In this study, we showed that artery wall hypoxia plays a role in causing IH formation through increased cellular proliferation. However, no clear mechanism has been proposed to explain how supplemental oxygen can alter artery wall pathology in cellular proliferation and IH.

One possibility may be that supplemental oxygen is responsible for increased free radical formation and subsequent oxidative stress at the anastomotic site prevents cellular proliferation. The presence of reactive oxygen species through the administration of hydrogen peroxide in cell culture has been shown to induce vascular smooth muscle cell apoptosis in a dose-dependent manner through the formation of hydroxyl radicals.[12] Similarly, radiation therapy, both external beam and catheter directed, has also been proposed as another therapy in preventing IH through free radical formation.[13]

Another possible effect of supplemental oxygen may actually be an antioxidant one. From previous studies, it has been shown that the placement of a PVG-to-artery anastomosis causes artery wall hypoxia. In rabbits without a PVG anastomosis (preoperative values), the artery wall oxygen tensions ranged from 50 to 60 mm Hg. In rabbits with a PVG anastomosis, the artery wall oxygen tensions ranged from 20 to 25 mm Hg.[10] After the administration of 40% supplemental oxygen, artery wall oxygen tensions were brought to supernormal levels of 100 to 200 mm Hg. Whether the increases in artery wall oxygen tension levels are more physiologic and reduce oxidative stress at the vascular anastomosis needs to be further elucidated. High concentrations of oxygen are what is considered to be the cause of oxidative stress, but it is also important to remember that low concentrations of oxygen can also cause oxidative stress through hypoxia and cell injury.

If, indeed, supplemental oxygen acts as an antioxidant at a PVG anastomosis, this would be consistent with other investigators' findings that antioxidants can prevent cellular proliferation and IH. For instance, α-tocopherol (vitamin E) has been found to inhibit vascular smooth muscle cells in vitro through the inhibition of the protein kinase C activity.[14] All-*trans*-retinoic acid (vitamin A analog), has also been implicated in reducing IH in the rabbit model.[15]

More recently, however, greater attention has been paid to the role of nitric oxide (NO) in the prevention of IH. NO is generated from the oxidation of the terminal nitrogen group on the amino acid, L-arginine. This oxidation process is facilitated by a single enzyme nitric oxide synthase (NOS). The overall reaction is as follows:

$$\text{L-arginine} + O_2 \xrightarrow{\text{NOS}} \text{NO} + \text{L-citrulline}$$

The precise role of NOS in the formation of NO is not completely understood. Nevertheless, several studies have shown that NO is an important modulator of vascular smooth muscle cell migration[16] and proliferation.[17] Researchers in several laboratories have induced NO concentrations with L-arginine supplementation, which has subsequently been shown to decrease IH.[4,18,19] Other proposed methods to increase NO synthesis have been to modulate the NOS enzyme through adenoviral vector transfection in vivo[20] or through ex vivo adenoviral-mediated NOS gene transfer to arterial or venous vessels.[6]

Either the addition of L-arginine through diet or NOS production through adenoviral transfection has been shown to decrease IH through increased NO production. Because oxygen plays an important role in the oxidation reaction to increase NO levels, it may be possible to achieve the same goals of increased NO production. The increased NO concentration, in turn, can decrease cellular proliferation and decrease IH through supplemental oxygen. Further investigation is necessary to determine whether oxygen is a rate-limiting substrate in NO production. On the basis of results presented here, the addition of oxygen does affect a change in vivo.

As mentioned previously, oxygen has been considered relatively safe, widely available, inexpensive, and much simpler to apply as therapy in preventing IH than that proposed by other investigators. Since the classic 19th century experiments by Paul Bert and Lorraine Smith demonstrating that oxygen in high concentrations was toxic to healthy mammalian lungs,[21] oxygen use for human use has been under investigation. A review of several prospective and retrospective studies has shown that multiday exposure to oxygen concentrations of 55% or less does not significantly impair pulmonary function in both normal healthy volunteers and human patients.[22]

In 1934, Richards and Barach[23] showed that exposure of 40% to 50% oxygen concentrations in patients with advanced heart failure and pulmonary disease and in two healthy volunteers for 5 days to 7 months showed little or no deleterious effects. Cardiac patients demonstrated an improved circulatory function, and in the pulmonary patients, improvement in arterial oxygen saturation was noted, as well as improved ambulatory activity. The two healthy volunteers experienced no adverse effects.[23] In 1927, Campbell and Poulton reported several years' work on the effects of continuous residence in a 40% oxygen chamber on patients with dyspnea. Most patients improved, as evidenced by less dyspnea, less cough and sputum, increased appetite, and weight gain.

As the oxygen concentrations were increased beyond 50% in human patients at varying amounts of time, varying degrees of pulmonary toxicity were seen. From these studies, a practical asymptote of an inspired P_{O_2} of 50% for pulmonary oxygen tolerance was developed.[22]

CONCLUSIONS

Forty percent of supplemental oxygen significantly reduces cellular proliferation and IH at the distal anastomosis of a PVG-to-artery anastomosis in the rabbit model. These findings suggest that the short-term administration of supplemental oxygen may be a simple, safe, and inexpensive method to control IH at a PVG-to-artery anastomosis.

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